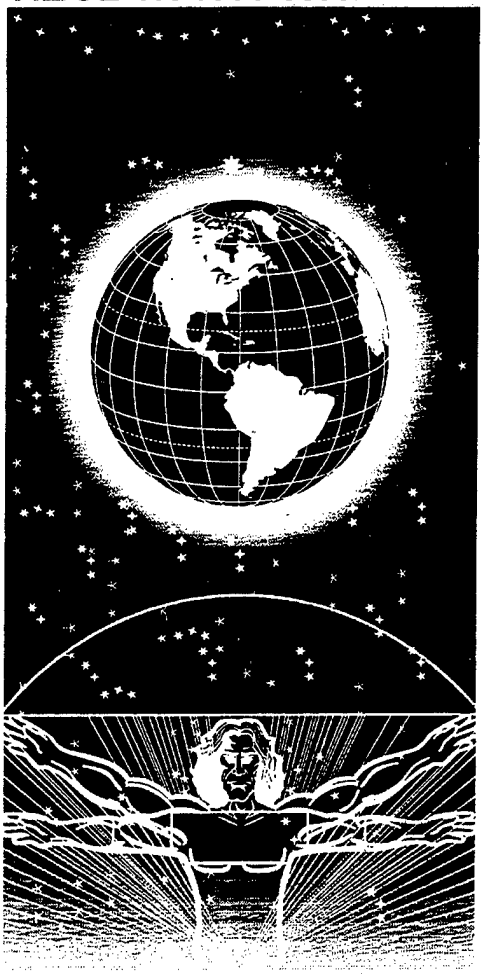
AL/OE-TR-1996-0077



UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

FREE RADICALS IN PRECISION CUT BOVINE TESTICLE SLICES: EFFECTS OF 1,3,5 TRINITROBENZENE MEASURED BY ELECTRON PARAMAGNETIC RESONANCE

Linda Steel-Goodwin
OCCUPATIONAL AND ENVIRONMENTAL HEALTH
DIRECTORATE TOXICOLOGY DIVISION
ARMSTRONG LABORATORY
WRIGHT-PATTERSON AFB, OH 45433-7400

John F. Wyman
MANTECH ENVIRONMENTAL TECHNOLOGY, INC.
DAYTON, OH 45437

Alasdair J. Carmichael
ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
BETHESDA, MD 20991

November 1996

Occupational and Environmental Health Directorate
Toxicology Division
2856 G Street
Wright-Patterson AFB OH 45433-7400

NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Air Force Armstrong Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Service
8725 John J. Kingman Rd., Ste 0944
Ft. Belvoir, Virginia 22060-6218

DISCLAIMER

This Technical Report is published as received and has not
been edited by the Technical Editing Staff of the Air Force Armstrong Laboratory.

TECHNICAL REVIEW AND APPROVAL

AL/OE-TR-1996-0077

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

STEPHEN R. CHANNEL, Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Armstrong Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1996	Interim Report - January 1994-March 1996

4. TITLE AND SUBTITLE
Free Radicals in Precision Cut Bovine Testicle Slices: Effects of 1,3,5 Trinitrobenzene Measured by Electron Paramagnetic Resonance

5. FUNDING NUMBERS
Contract
PE 61102F
PR 2312
TA 2312A2
WU 2312A202

6. AUTHOR(S)
L. Steel-Goodwin, J.F. Wyman, and A.J. Carmichael

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AL/OE-TR-1996-0077

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Precision cut tissue slices were prepared from bovine testes and incubated at 37 degrees C in Wymouth's media supplemented with the spin trap N-tert-butyl-*a*-nitrone or dimethyl sulfoxide. Slices were exposed for 0 to 1h with 0-1000 uM 1,3,5 trinitrobenzene (TNB). Radicals were detected in testis after glutathione depletion with diethylmaleate in control, TNB-treated and superoxide-treated slices. The hyperfine coupling constant of the TNB-induced PBN-radical adducts was aN=1.629 mT and aHB=0.353 mT. Analysis of variance showed more radicals were trapped in the TNB-treated slices 100 uM-1000uM when compared to controls ($P<0.0001$). First order regression analysis of the dose response curve was $y=0.04x+23$($r2=0.66$, $n=30$). This data shows the *in vitro* slice technique can be used to measure radicals induced by TNB in bovine testicles.

14. SUBJECT TERMS
1,3,5 Trinitrobenzene (TNB) testis tissue slice
free radicals superoxide EPR/spin trapping

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

PREFACE

This is one of a series of technical reports describing the results of the electron paramagnetic resonance laboratory data conducted at the Occupational and Environmental Health Directorate, Toxicology Division and at the Armed Forces Radiobiology Research Institute, Bethesda MD. The samples were prepared by and the spectra generated by Dr. John F. Wyman when he was supported by the US Army and the Department of the Air Force Contract No 33615-90-C-0532 from 1994-1996. Collaborative interpretation of the spectra was sponsored by the Air Force Office of Scientific Research Environmental Initiative Program work unit 2312A202 and AFRRI Work Unit 04630. Lt Col Terry A. Childress served as Contract Technical Monitor for the US Air Force, Armstrong Laboratory, Toxicology Division.

The authors wish to thank Mr. Larry Landes and Landes Meats Inc. for their donation of bull testicles and Dr J Lipscomb for his advice on glutathione reactions. The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

C	Centrigrade
DEM	Diethylmaleate
DMSO	Dimethyl sulfoxide
EPR	Electron Paramagnetic Resonance spectrometer
g	Gram
h	Hour
K	Kelvin
L	Liter
mg	Milligram
mm	millimeter
PBN	N-tert-butyl-α-nitrone
T	Tesla
TNB	Trinitrobenzene
µl	microliter

SECTION 1

INTRODUCTION

1, 3, 5 Trinitrobenzene (TNB) has been identified as a contaminant along with other munitions in soil and ground water at numerous U.S. Army installations (Walsh and Jenkins, 1992). TNB is a nitroaromatic produced as a biproduct of the manufacture of trinitrotoluene. There are a number of studies reporting damage to the testes in experimental animals by nitroaromatics (Foster, 1992). For example, in experimental animals nitrobenzene (Bond et al, 1981), dinitrobenzene (Cody et al., 1981) and trinitrotoluene (Levine et al., 1981) cause testicular damage. Rats exposed to TNB in diet had depleted sperm counts and degeneration of seminiferous tubules (Kinkead et al, 1995). We found no reports that TNB is a human reproductive toxicant, but there are reports that male workers exposed daily to organic solvents such as benzene, toluene or dinitrotoluene and toluene diamine have oligoasthenoteratozoospermia (Radike, 1985). Oligoasthenoteratozoospermiais a condition of reduced sperm count and those sperm present are immobile and abnormal looking.

In vivo rat studies (Kinkead et al., 1995) provided limited information on the mechanism of TNB toxicity, so a precision cut testis slice model was developed. Because of the relatively low amount of intertubular connective tissue, slices from rat testes could not be prepared (Wyman et al., 1996). Bull and dog testes have sufficient intertubular connective tissue to allow preparation of proper slices. The evaluation of TNB toxicity was performed using bull testes because this tissue was easily obtained through a local meat processing company.

Using this *in vitro* slice model, histology preparations of bull testes slices exposed to TNB showed increased necrosis of the germinal epithelium compared to control slices (Wyman et al., 1996) which was a finding consistent with that observed in previous *in vivo* exposures (Kinkead, et al., 1995)

In the presence of diethyl maleate (DEM), testicular slices also had increased lipid peroxidation as measured by TBARS assay. DEM is know to deplete endogenous levels of the antioxidant, glutathione. TNB, in the presence of DEM, blocked the increase in lipid peroxidation in testicular slices (Wyman, et al., 1996).

Lipid peroxidation is a chain reaction which results from the abstraction of a hydrogen atom from a methylene group by a reactive species (Gutteridge, 1995a). Examples of species which can abstract the hydrogen and initiate lipid peroxidation are the hydroxyl radical, alkoxyl radical and peroxyl radical (Gutteridge, 1995b). In the testis slice study TBARS depression was dose-dependent (Wyman et al., 1996) and suggested TNB or its decomposition products altered free radical levels or even generated radicals which did not have enough energy to abstract a hydrogen atom necessary for lipid peroxidation.

To test this hypothesis we used electron paramagnetic resonance (EPR) to detect free radicals. It was reasonable to expect that radical adducts generated by testicular slices may be trapped and detected by EPR using spin traps. A number of spin traps have been used to detect radicals in biological slices (Steel-Goodwin et al 1992, Myers et al 1994 and Steel-Goodwin et al 1995). This technique involves the use of nitroso compounds (Lagercratz, 1971) and nitrones (Janzen, 1971) which react with short-lived

radicals to produce more stable adducts (Buettner, 1982). The spectral information contained in the adduct is often less than that of the parent radical (Joshi and Yang, 1981).

Free radical chemistry of the testis was studied to elucidate TNB toxicity effects because: *one*, nitroaromatic compounds have been reported to act through free radical pathways (Romero, et al., 1995); *two*, cells in the seminiferous tubules are very sensitive to free radical damage and are protected by a blood-testis barrier; *three*, the seminiferous tubules have very low levels of catalase (Burhley and Ellis, 1973) which reacts very rapidly with hydrogen peroxide and is important when glutathione peroxidase or reduced glutathione levels are unavailable to remove hydrogen peroxide (Rice Evans et al., 1991) and *four*, unique histones are present in the testes which may act as free radical scavengers (Zenick and Clegg, 1986). Other nitrocompounds have been reported to have cytotoxic effects in testes because their reduction catalyses the formation of superoxide radicals (Decompo et al 1981). Superoxide radicals cannot initiate lipid peroxidation and neither can hydrogen peroxide (Gutteridge, 1995b) and this could explain the lipid peroxidation effects observed in this study (Wyman et al., 1996). The possibility of TNB generated radicals was investigated using EPR/spin trapping.

SECTION 2

MATERIALS & METHODS

Chemicals: 1, 3, 5-Trinitrobenzene (TNB) was obtained from Dr. Gunda Reddy, Army Biomedical Research and Development Laboratory, Ft. Detrick, Fredrick, MD. All other chemicals were research grade obtained from Sigma or Aldrich.

Sample Analysis: Bovine testicles were donated by Landes Meats, Inc., Dayton, OH. The tissue was prepared and sliced as described previously (Wyman et al 1996) and placed in 1.7 ml incubation medium. The incubation medium (pH 7.4 ± 0.1) was Waymouth's supplemented with 10% fetal calf serum (Gibco), gentamycin (84mg/ml), L-glutamine (3.5 mg/ml) and sodium bicarbonate (2.4 mg/ml). A saturated solution of potassium superoxide (Aldrich Chemical Co) was prepared in 250 ul dimethylsulfoxide (DMSO, Aldrich Chemical Co) and 5 ul added in experiments involving superoxide. TNB was dissolved in DMSO and added to the media to give final concentrations of 0, 50, 100, 500, or 1000 uM as described previously (Wyman et al 1996). The spin trap, N-tert-butyl-α-phenyl nitrone (PBN, Aldrich Chemical Co) was weighed and dissolved in 500 µl DMSO just prior to addition to the incubation media so that the final concentration of PBN in the incubate was 10 mM. All incubations were carried out in media supplemented with diethylmaleate (DEM, Wyman et al., 1996) unless otherwise stated. The slices were incubated in glass scintillation vials and throughout the experiment they were protected from ultraviolet and fluorescent light sources. At the end of the incubation period 0-60 min, the testes slice was homogenized in the media, frozen in liquid nitrogen (77K) and lyophilized. The lyophilized media was rehydrated in 100µl double distilled water,

vortexed and 20 μl was drawn into a glass capillary tube, sealed, and placed in the cavity of an EMS EPR Analyzer (Bruker Instruments, Billerica, MS). The EPR instrument was calibrated with pitch (Bruker, Instruments, MS) and operated, unless otherwise noted, at a magnetic field set at 3.475 mT, modulation amplitude 0.16mT, time constant 1310.72 ms, scan time 167.77 ms, scan range 0.6mT and temperature 25°C. Hyperfine values were determined by direct measurement from the spectra and computer simulated.

Statistical Analysis: Analysis of variance was performed using Design Ease® and regression analysis was determined by Sigma Stat® and Sigma Plot® statistical programs.

SECTION 4

RESULTS & DISCUSSION

In the control bull testes there were radical adducts detected when the slices were incubated with DMSO alone and with the PBN spin trap. This suggests either the radicals detected are the result of cell damage or that the slices produce radicals as part of their normal metabolism. Cell viability data and TBARS analysis support the latter explanation (Wyman et al, 1996). Figure 1A shows the typical spectrum of PBN radical adducts in lyophilized slices following 10 min incubation. In the presence of TNB, radicals were also detected, Figure 1B. Control media gave only a broad signal, Figure 1C.

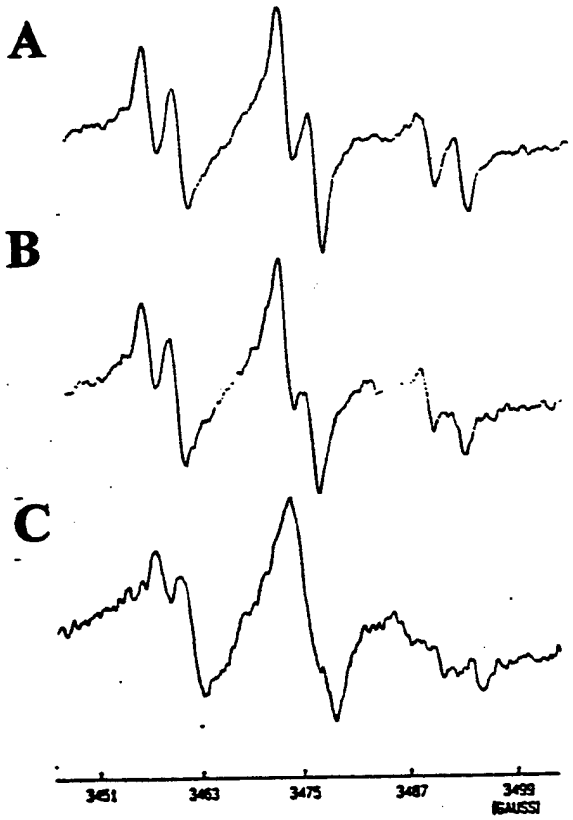


Figure 1. ***A. Spectrum of control testis slice. B. Spectrum of TNB-treated testis slice C. Control media no testis***

The radicals induced by TNB are shown in Figure 2A. When this spectrum was computer simulated the hyperfine coupling constants were a_N = 1.629mT and a_H^{β}= 0.353mT, Figure 2B. TNB alone without slices had a broad signal, Figure 2C, which was similar to media alone, Figure 1C.

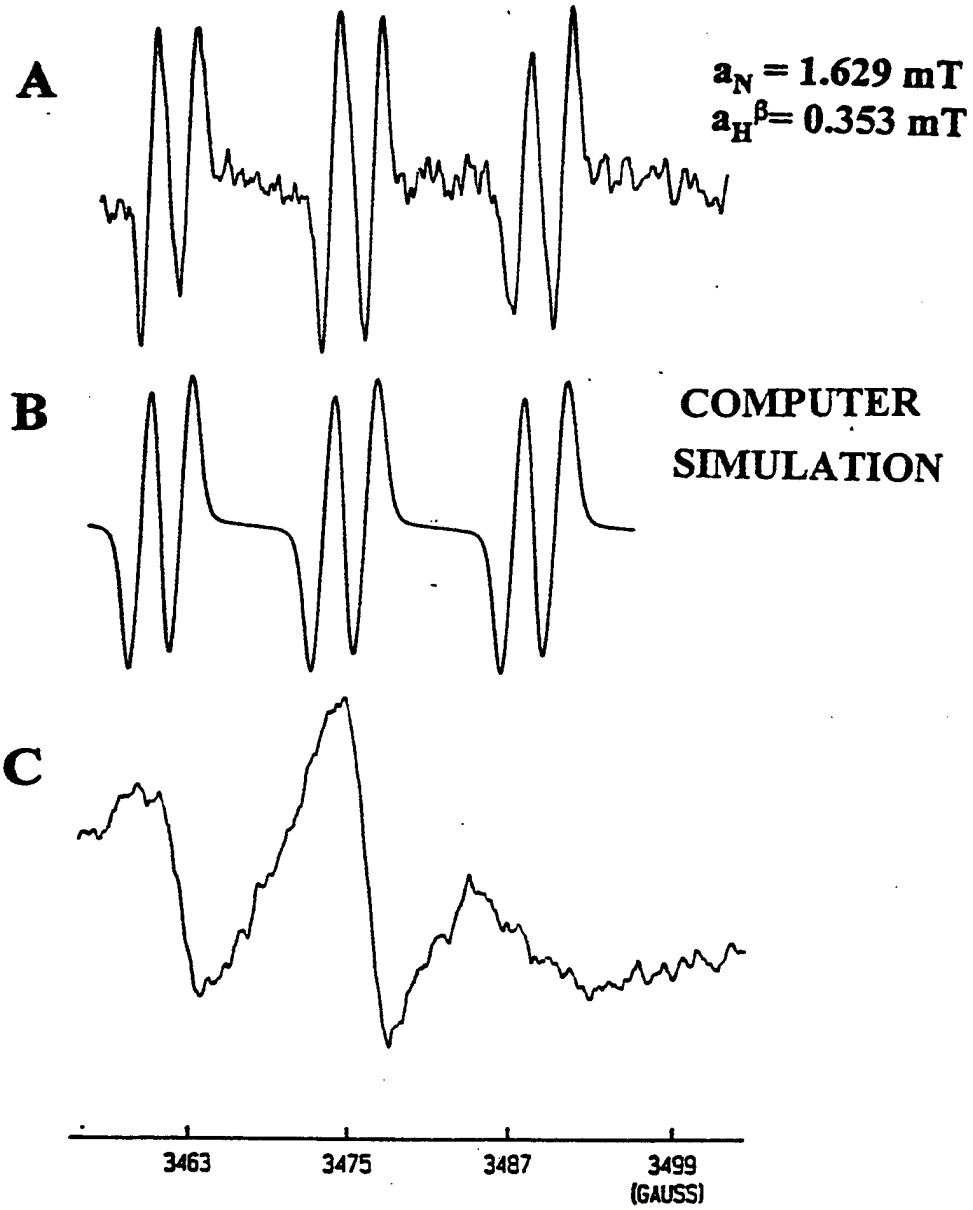


Figure 2 A ***Spectrum of TNB induced radicals in testis slices*** **B.** ***Computer simulated spectrum of A.*** **C** ***Spectrum of media with TNB and no testis.***

Trinitrobenzene (TNB) is a nitroaromatic which we hypothesised was metabolised similar to dinitrobenzenes and would produce an EPR signal in DMSO, the solvent used to dissolve TNB and the spin trap, PBN. Possible radical adducts are shown in Figure 3.

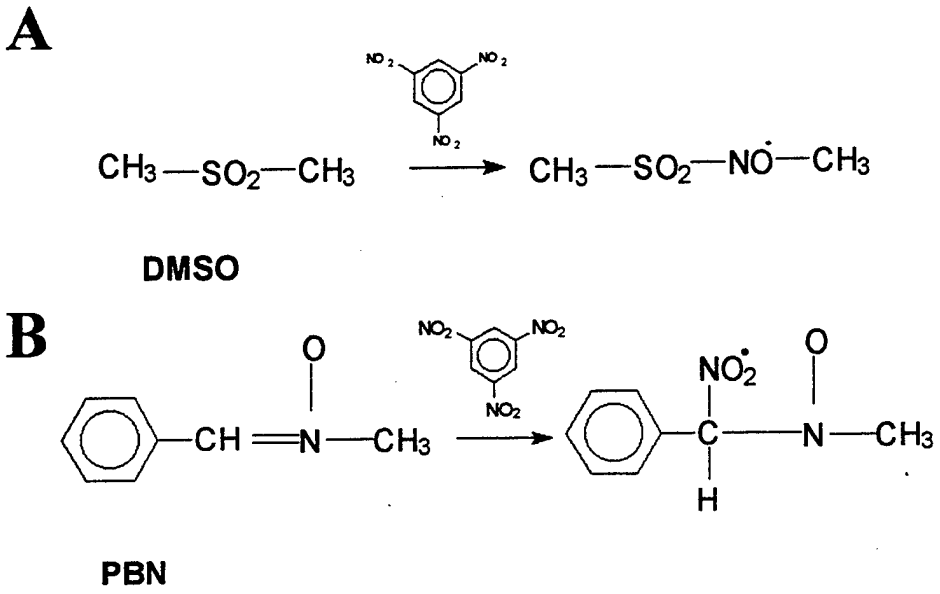


Figure 3 ***Reactions of DMSO and PBN with decomposition products of TNB.***

Experimentally radicals were detected in DMSO alone, as well as with PBN, Figures 1& 2. DMSO is a classical solvent for studying the EPR signal of nitrobenzene (Rice Evans et al., 1991) and recently DMSO has been used to trap peroxynitrite-induced radicals formed by reaction of nitric oxide with superoxide (Carmichael and Steel-Goodwin, 1994). Figure 4 shows the radicals detected in DMSO in the presence of slices alone, Figure 4A and with TNB, Figure 4B. Similar spectra were observed in both the control and TNB-treated slices. Control media had a broad signal, Figure 4C.

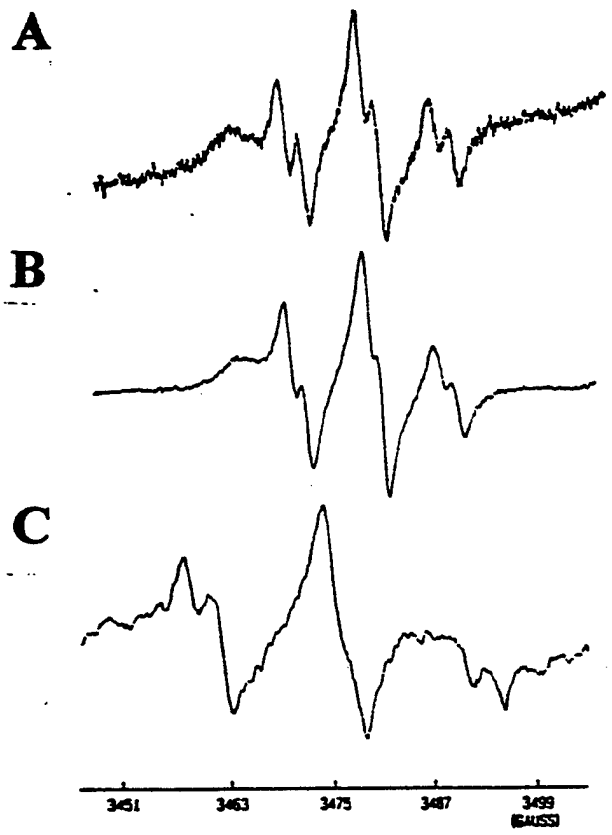


Figure 4 A *Spectrum of testis slices incubated in DMSO.* B. *Spectrum of testis slices incubated with TNB in DMSO.* C. *Spectrum of control media.*

To determine if the cytotoxic effects of TNB in testis slices was because TNB-reduction catalyzes the formation of superoxide radicals (Decampo et al, 1981), experiments were repeated with and without dimethylmaleate (DEM) in the incubate in the

presence of superoxide and the results were compared with TNB-exposed samples, treated similarly, Figure 5.

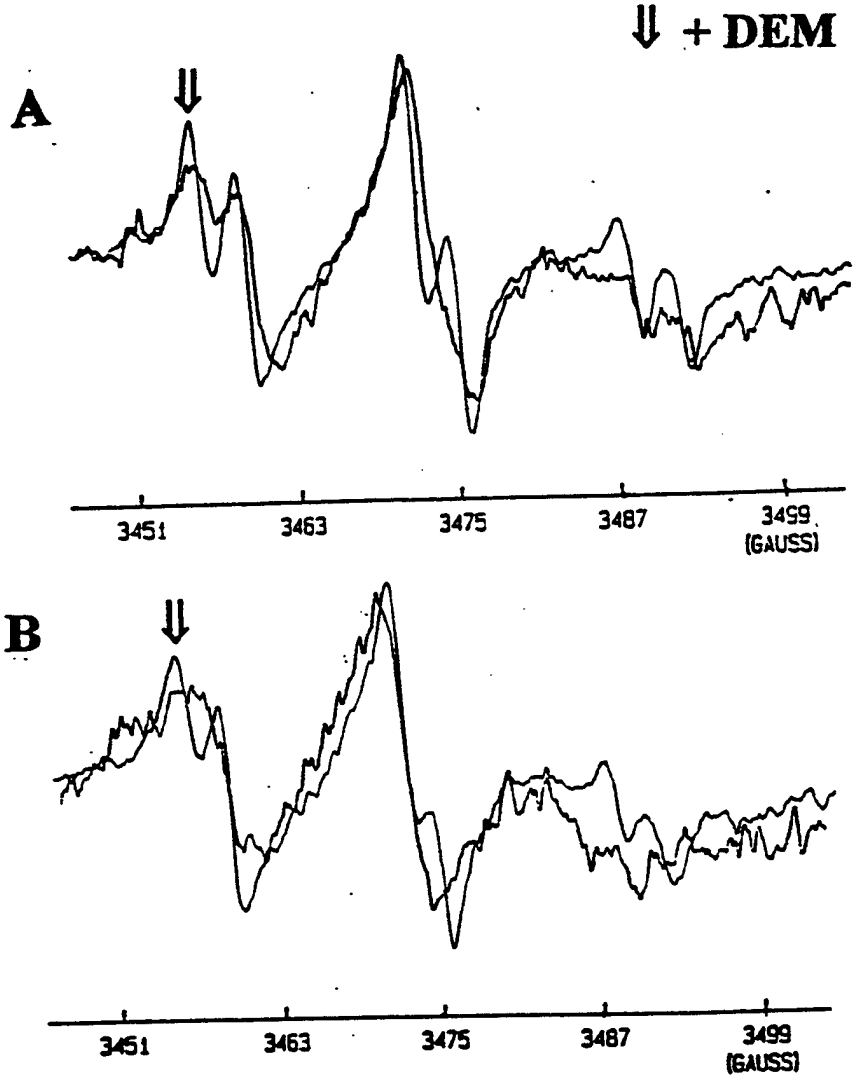


Figure 5 A ***The spectra of control slices exposed to superoxide with and without DEM.*** **B** ***The spectra of TNB treated slices exposed to superoxide with and without DEM.***

Slices of testis incubated without DEM had a broad spectrum, Figure 5A even though superoxide was added. The typical PBN-radical adducts shown in Figure 1A were

detected in the presence of DEM and superoxide, Figure 5A. The TNB-treated slices, Figure 5B were similar to the controls, Figure 5A. In the presence of DEM and superoxide, a triplet of doublets was detected. With no DEM in the incubate the signal was similar to control TNB media, Figure 2C. DEM is a commonly used reagent for tissue glutathione depletion. DEM conjugates with glutathione and this raises the baseline of TBARS (Wyman unpublished data). DEM presence in the incubate probably alters availability of NADPH, an electron donor. Well resolved EPR spectra are known for many of the dinitrobenzenes where superoxide reacts to yield corresponding nitrophenols (Poupko and Rosenthal, 1973), but we found no literature on the effects of superoxide with TNB. The present experimental data does suggest testes slices incubated *in vitro* for 0-60 min with TNB releases free radicals, Figures 1, 2, 4 & 5.

EPR can be used to determine the quantity of radicals by measurement of the intensity of the EPR signal. The EPR intensity measured in the testes slices after incubation for 60 min is shown in Figure 6. By analysis of variance there was more radicals detected in slices with TNB compared to controls ($r^2 = 0.73$, $F= 27.47$, $P< 0.0001$), Appendix A. Linear regression analysis of the plot shown in Figure 6 is given in Appendix B. Further studies are necessary to measure the actual number of radicals detected with known radical standards, such as stable spin labels which have been used successfully for measuring TCE radicals (Steel-Goodwin et al 1994).

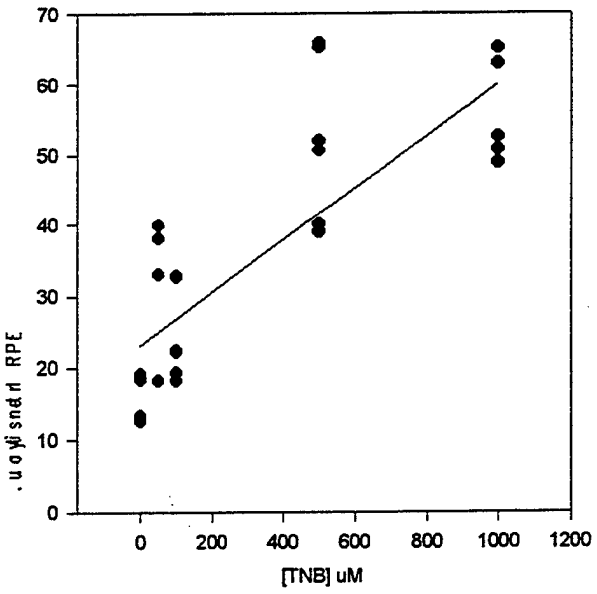


Figure 6. ***EPR intensity of slices after incubation for 1h in 0-1 mM TNB.***

Nitroaromatics are believed to be metabolized by nitroreductases by a mechanism which uses NADPH as the electron donor and molecular oxygen which leads to production of the superoxide radical and regeneration of the nitrocompound (Decompo et al 1981, Mason & Josephy 1985, Biaglow et al 1986, Mason 1990, Romero et al 1995). DEM depletes glutathione in the testis slices and raises the TBARS baseline so it is reasonable to suggest that hydroxyl radicals are generated in the slices and these are involved in the TNB-induced testicular damage. Equations 1-8 shown below are the possible events. Cells are equipped with NADPH-dependent glutathione reductase to reduce the oxidized glutathione, Equation 3. Although superoxide dismutase is the major antioxidant enzyme in the testis, Equation 4, NADPH is also important in reducing

superoxide to hydrogen peroxide, Equation 6. NADPH is also important in nitroreductase activity, Equation 8.

$GSH + H_2O_2$	$\rightarrow$	$GS\text{-}SG + 2H_2O$	(1)
$LOOH$	$\rightarrow$	$GS\text{-}SG + LOH + H_2O$	(2)
$GS\text{-}SG$	$\rightarrow$	$2GSH$	(3)
$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+$	$\rightarrow$	$H_2O_2 + O_2$	(4)
$O_2^{\cdot-} + H_2O_2$	$\rightarrow$	$OH^{\cdot} + OH^- + O_2$	(5)
$O_2^{\cdot-} + NADPH$	$\rightarrow$	$H_2O_2 + NADP^{\cdot}$	(6)
$OH^{\cdot} + NADPH$	$\rightarrow$	$H_2O + NADP^{\cdot}$	(7)
$NADP^{\cdot} + TNB$	$\rightarrow$	$NADP^+ + TNB^{\cdot-}$	(8)

Further studies are necessary to elucidate the mechanism of DEM action in the generation of radicals in the slices especially the role of the unique histones present in the testis. These histones can act as substrates for cysteine. The presence of TNB in the incubate upsets the delicate balance between oxygen and nitrogen centered radicals in the seminferous tubules. Oxygen centered radicals are necessary for cell division in the germ cells and their levels are normally under strict control by nitrogen centered radicals. Elevation of oxygen centered radicals may explain the pathological damage to both the germ cells and sertoli cells in the slices incubated with TNB (Wyman et al 1996). The decomposition products of TNB have still to be identified, as well as the radical species which is induced by TNB and trapped by DMSO and PBN, Figure 3. However, the role of free radicals in TNB toxicity is particularly timely and this study in the testis compliments new information on the role of cysteine residues in hemoglobin to carry the free radical NO (Jai et al 1996) and the clinical reports that nitroaromatics and pharmaceuticals with similar chemical structure to TNB cause methemoglobinemia even when topically applied (Dinneen et al 1994).

REFERENCES

Biaglow, J.E, M.E. Varnes, L. Roizen-Torle, E.P. Clark, E.R. Epp, M. B. Astor, E. J. Hall 1986 Biochemistry of reduction of nitro heterocycles. Biochem Pharmacol 35: 77-90.

Bond, J.A., Chism, J.P., Rickert, D.E. and Popp, J.A. Induction of hepatic testicular lesions in Fischer F-344 rats by single oral doses of nitrobenzene. Fundam. Appl. Toxicol. 1, p389, 1981.

Buettner, G.R. 1982 The spin trapping of superoxide and hydroxyl radicals . In Superoxide Dismutase Ed L.W. Oberley Vol II Chapter 4 pp 63-81. CRC Press, Inc Boca Raton, FL.

Burhley, L.E. and Ellis, L.C. 173 Fertil. Steril. 24: 956-961

Carmichael, A.J. and L. Steel-Goodwin 1994 Decomposition of peroxynitrite studied by EPR/spin trapping J Free Rad in Biology and Medicine 2 3: Q5.

Cody, T.E., Witherup, S., Hastings, L., Stemmer, K., and Christain, R.T. 1,3-Dintrobenzene: toxic effects in vivo and in vitro. J. Toxicol. Environ. Health 7, 829, 1981.

Decompo, R., R.P. Mason, C. Mottley, and R.P.A. Mupiz 1981 Generation of free radicals induced by nifurtimox in mammalian tissues J Biol Chem 256 21: 10930-10933.

Dinneen, S.F., D.N. Mohr and V.F. Fairbanks 1994. Methemoglobinemia from topically applied anaethetic spray. Mayo Clin Proc 69: 886-888.

Foster, P.M.D. 1992 The sertoli cell. In Reversibility in Testicular Toxicity Assessment Chapter 2 Ed. A.R. Scialli and E.D.Clegg CRC Press Inc, Boca Raton, FL.

Gutteridge, J.M.C. 1995a Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 41, 12B, 1819-1828.

Gutteridge, J.M.C. 1995b Lipid peroxidation: some problems and concepts. In Halliwell B, ed. Oxygen radicals and tissue injury, Bethesda MD: FASEB, 1988: 9-19.

Jai, L, S. Stamler 1996 Nature 380, 221

Janzen, E.G. 1971 Spin trapping Acc Chem Res 4, 31.

Joshi, A. and G.C. Yang 1981 Spin trapping of radicals generated in the uv photolysis of alkyl disulfides J Org Chem 46: 3736-3738.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and G.B. Marit (1995) Reproductuve toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. Toxicol. Indust. Health 11 (3) 309-323.

Lagercratz, C.J. 1971 Spin trapping of some short-lived radicals by the nitroxide method. J. Phys Chem 75 3466-3475

Levine, B.S., Furedi, E.M., Gordon, D.E., Lish, P.M., and Barkley, J.J. Subchronic toxicology of trinitrotoluene in Fisher F-344 rats Toxicology 32, 253, 1984.

Mason, R.P., Josephy, P.D. 1985 Free radical mechanism of nitroeductase: In: Rickert, D.E. ed. The toxicity of nitroaromatic compounds. New York: Hemisphere Publishing Corp pp 121-140.

Mason, R.P. 1990 Redox cycling of radical anion metabolites of toxic chemicals and drugs and the Marcus theory of electron transfer. Environ. Health Perspect. 87: 237-243

Myers, Jr., L.S., A.J. Carmichael, and T.C. Pellmar 1994 Radiation chemistry of the hippocampal brian slice 1994 Adv. Space Res. Vol 14 (10) 453-456.

Poupko, R and I. Rosenthal 1973 Electron transfer interations between superoxide ion and organic compounds. J. Phys Chem 77: 1722-1724.

Radike, M. Reproductive Toxicology Chpter16 In Industrial Toxicolgy Ed. Williams, P.L. and J.L. Burson 1985, Van Nostrand Reinhold.

Romero, I.A.., T. Lister, H.K. Richards, M.P. Seville, S.P. Wylie, D.E. Ray 1995 Early metabolic changes during m-dinitrobenzene neurotoxicity and the possible role of oxidative stress. Free Rad. Biol and Med 18 (2) 311-319.

Rice-Evans, C.A., A.T. Diplock, M.C.R. Simons 1991 In Techniques in free radical research Ed R.H. Burdon and P.H. van Knippenburg Elsevier Science Publishing Co., NY.

Steel-Goodwin L, Arroyo CM, Gray BH and Carmichael AJ. EPR detection of nitric oxide dependent spin adducts in mouse jejunum. In Biology of Nitric Oxide Vol 2 Enzymology, Biochemistry and Immunology, ED S. Moncada, MA Marletta, JB Hibbs Jr and EA Higgs Portland Press, London pp 80-84, 1992

Steel-Goodwin L, Pravecek TL and Carmichael AJ Trichloroethylene: An EPR Study Free Rad Biol Med 1994: (2) 3 Q10.

Walsh, M.E. and T.F. Jenkins 1992 Identification of TNT transformation products in soil. Special Report 92-16. U.S. Army Corps of Engineers, Cold Regions Research & Engineering Laboratory.

Wyman, J.F., J.S. Eggers, C.D. Flemming 1996 Preparation of bovine testicular tissue slices: Evaluation of 1,3,5-trinitrobenzene toxicity. Reproduc. Toxicol. (In revision).

Zenick H and E.D. Clegg 1989 Assessment of male reproductive toxicity: a risk assessment approach. In Principles and Methods of Toxicology A.W. Hayes Ed., 2nd ed. pp 275-309 Raven Press, New York.

SECTION 5

APPENDIX A

Data was analyzed using the Design Ease® for Windows from Stat-Ease Inc. Minneapolis, MN.

1. Design Layout

Std	Den Id	Run	Block	TNB au Factor	EPR Response
1	1	19	1	1000	62.88
2	1	23	1	1000	65.14
3	1	5	1	1000	48.76
4	1	16	1	1000	52.41
5	1	13	1	1000	48.90
6	1	2	1	1000	50.67
7	2	17	1	500	50.56
8	2	30	1	500	51.86
9	2	26	1	500	38.99
10	2	8	1	500	39.95
11	2	15	1	500	65.33
12	2	7	1	500	65.85
13	3	28	1	100	32.66
14	3	27	1	100	32.79
15	3	29	1	100	22.04
16	3	24	1	100	22.37
17	3	20	1	100	18.15
18	3	1	1	100	19.18
19	4	6	1	50	32.91
20	4	21	1	50	33.04
21	4	9	1	50	38.09
22	4	22	1	50	39.82
23	4	14	1	50	18.12
24	4	25	1	50	18.12
25	5	4	1	0	12.67
26	5	11	1	0	12.48
27	5	12	1	0	12.76
28	5	18	1	0	18.29
29	5	10	1	0	13.34
30	5	3	1	0	19.08

```
***** BRUKER  EMS104 *****

DATE      : 24-APR-1996
TIME      : 14::9:00
OPERATOR  :
SAMPLE    : s30um
UNITS     :
COMMENT   :

MP  :   25.06
HSW :  100.00
RMA :    4.02
RCT :   10.49
RTC :   20.48
RRG :      30
ROF :       0
RPH :       0
HCF :    0.30
JNS :       4
SH  :      20

**************************

   1:        62.88
   2:        65.14
   3:        48.76
   4:        52.41
   5:        48.90
   6:        50.67
   7:        50.56
   8:        51.86
   9:        38.99
  10:        39.95
  11:        65.33
  12:        65.95
  13:        32.66
  14:        32.79
  15:        22.04
  16:        22.37
  17:        18.15
  18:        19.18
  19:        32.91
  20:        33.04
  21:        38.09
  22:        39.82
  23:        18.12
  24:        18.12
  25:        12.67
  26:        12.48
  27:        12.76
  28:        18.29
  29:        13.34
  30:        19.08
  31:        10.04
  32:        22.07
  33:        45.68
  34:        65.22
  35:        98.75
```

2. Analysis of EPR Data

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F VALUE	PROB > F
MODEL	7363.3671	4	1840.84	27.47	< 0.0001
RESIDUAL	1675.3610	25	67.01		
COR TOTAL	9038.7281	29			

ROOT MSE	8.1862	R-SQUARED	0.81
DEP MEAN	35.2403	ADJ R-SQUARED	0.78
C.V. %	23.2297	PRED R-SQUARED	0.73

Predicted Residual Sum of Squares (PRESS) = 2412.52

TREATMENT MEANS (ADJUSTED, IF NECESSARY)

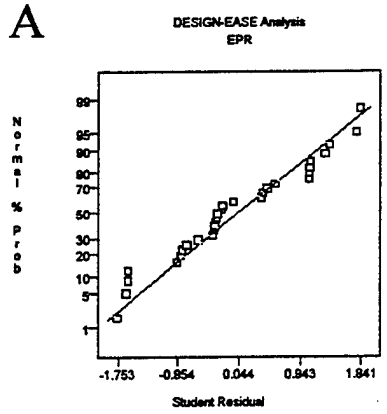
	ESTIMATED MEAN	STANDARD ERROR
1000	54.7933	3.3420
500	52.0900	3.3420
100	24.5317	3.3420
50	30.0167	3.3420
0	14.7700	3.3420

Treatment	MEAN DIFFERENCE	DF	STANDARD ERROR	t FOR H0 COEFFICIENT=0	PROB > |t|
1 vs 2	2.70	1	4.726	0.572	0.5724
1 vs 3	30.26	1	4.726	6.403	< 0.0001
1 vs 4	24.78	1	4.726	5.242	< 0.0001
1 vs 5	40.02	1	4.726	8.468	< 0.0001
2 vs 3	27.56	1	4.726	5.831	< 0.0001
2 vs 4	22.07	1	4.726	4.670	< 0.0001
2 vs 5	37.32	1	4.726	7.896	< 0.0001
3 vs 4	-5.48	1	4.726	-1.161	0.2568
3 vs 5	9.76	1	4.726	2.065	0.0494
4 vs 5	15.25	1	4.726	3.226	0.0035

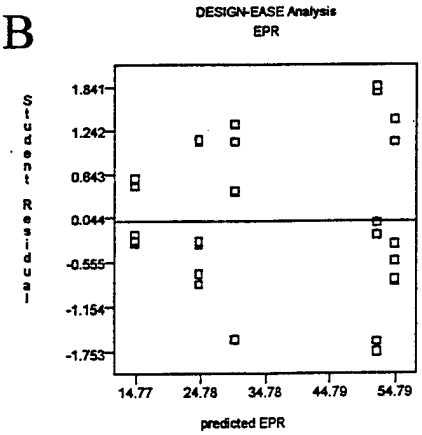
OBS ORD	ACTUAL VALUE	PREDICTED VALUE	RESIDUAL	STUDENT LEVER	COOK'S RESID	OUTLIER DIST	T VALUE	RUN ORD
1	62.88	54.79	8.087	0.167	1.082	0.047	1.086	19
2	65.14	54.79	10.347	0.167	1.385	0.077	1.412	23
3	48.76	54.79	-6.033	0.167	-0.807	0.026	-0.802	5
4	52.41	54.79	-2.383	0.167	-0.319	0.004	-0.313	16
5	48.90	54.79	-5.893	0.167	-0.789	0.025	-0.782	13
6	50.67	54.79	-4.123	0.167	-0.552	0.012	-0.544	2
7	50.56	52.09	-1.530	0.167	-0.205	0.002	-0.201	17
8	51.86	52.09	-0.230	0.167	-0.031	0.000	-0.030	30
9	38.99	52.09	-13.100	0.167	-1.753	0.123	-1.834	26
10	39.95	52.09	-12.140	0.167	-1.625	0.106	-1.683	8
11	65.33	52.09	13.240	0.167	1.772	0.126	1.856	15
12	65.85	52.09	13.760	0.167	1.841	0.136	1.940	7
13	32.66	24.53	8.128	0.167	1.088	0.047	1.092	28
14	32.79	24.53	8.258	0.167	1.105	0.049	1.110	27
15	22.04	24.53	-2.492	0.167	-0.333	0.004	-0.327	29
16	22.37	24.53	-2.162	0.167	-0.289	0.003	-0.284	24
17	18.15	24.53	-6.382	0.167	-0.854	0.029	-0.849	20
18	19.18	24.53	-5.352	0.167	-0.716	0.021	-0.709	1
19	32.91	30.02	2.893	0.167	0.387	0.006	0.380	6
20	33.04	30.02	3.023	0.167	0.405	0.007	0.398	21
21	38.09	30.02	8.073	0.167	1.080	0.047	1.084	9
22	39.82	30.02	9.803	0.167	1.312	0.069	1.332	22
23	18.12	30.02	-11.897	0.167	-1.592	0.101	-1.645	14
24	18.12	30.02	-11.897	0.167	-1.592	0.101	-1.645	25
25	12.67	14.77	-2.100	0.167	-0.281	0.003	-0.276	4
26	12.48	14.77	-2.290	0.167	-0.306	0.004	-0.301	11
27	12.76	14.77	-2.010	0.167	-0.269	0.003	-0.264	12
28	18.29	14.77	3.520	0.167	0.471	0.009	0.464	18
29	13.34	14.77	-1.430	0.167	-0.191	0.001	-0.188	10
30	19.08	14.77	4.310	0.167	0.577	0.013	0.569	3

3. Diagnostic and Interpretation Graphs

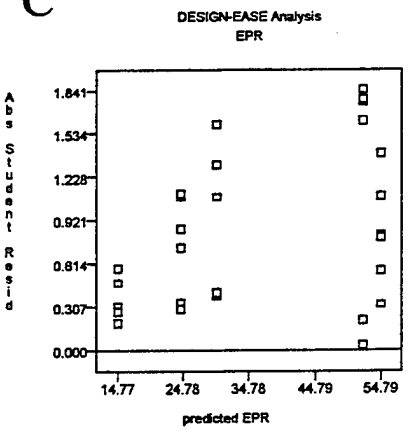
A



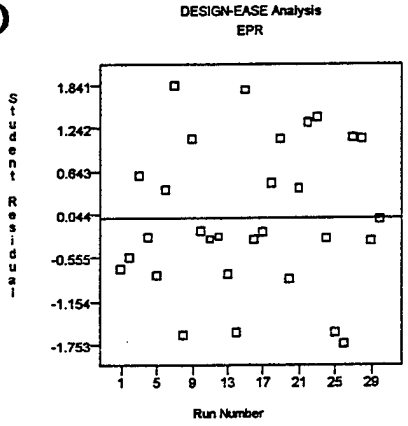
B



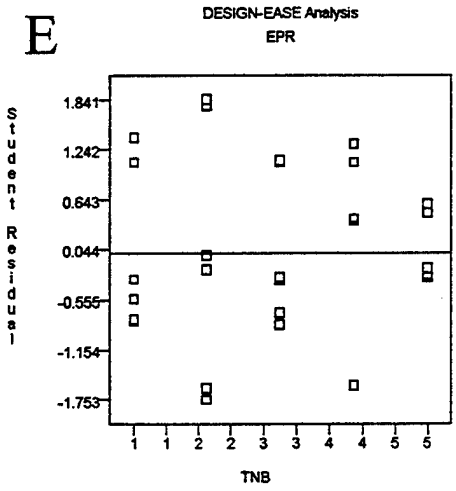
C



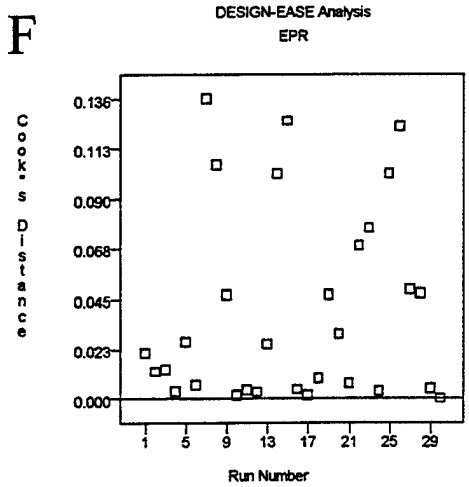
D



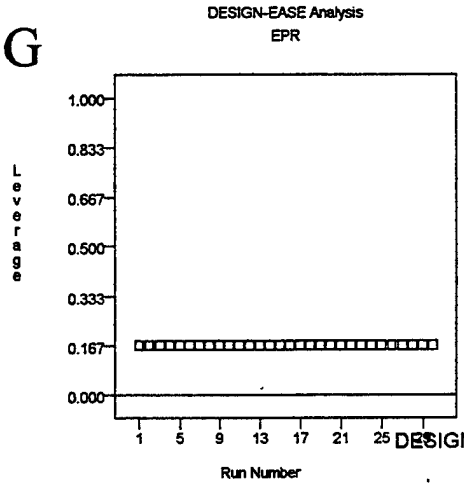
E


DESIGN-EASE Analysis
EPR
Student Residual
1.841
1.242
0.643
0.044
-0.555
-1.154
-1.753
1 1 2 2 3 3 4 5 5
TNB

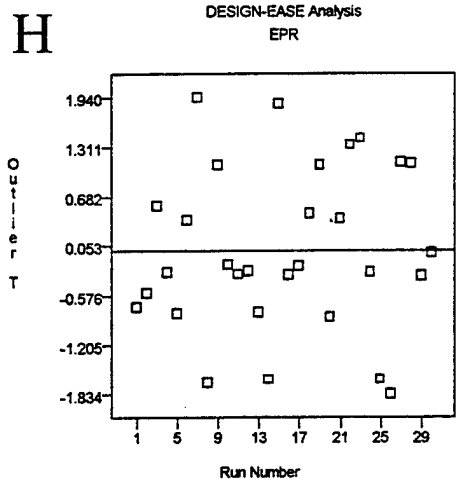
F


DESIGN-EASE Analysis
EPR
Cook's Distance
0.136
0.113
0.090
0.068
0.045
0.023
0.000
1 5 9 13 17 21 25 29
Run Number

G

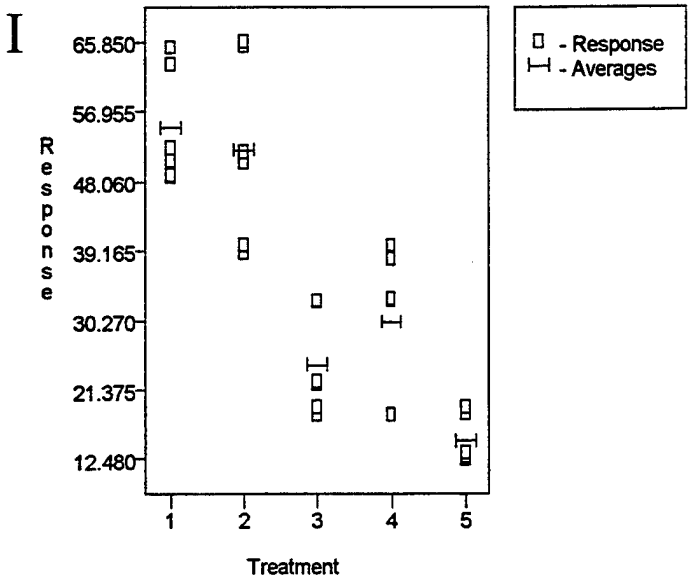

DESIGN-EASE Analysis
EPR
Leverage
1.000
0.833
0.667
0.500
0.333
0.167
0.000
1 5 9 13 17 21 25
Run Number

H


DESIGN-EASE Analysis
EPR
Outlier T
1.940
1.311
0.682
0.053
-0.576
-1.205
-1.834
1 5 9 13 17 21 25 29
Run Number

DESIGN-EASE Analysis
EPR

I


Response
65.850
56.955
48.060
39.165
30.270
21.375
12.480
1 2 3 4 5
Treatment
- Response
- Averages

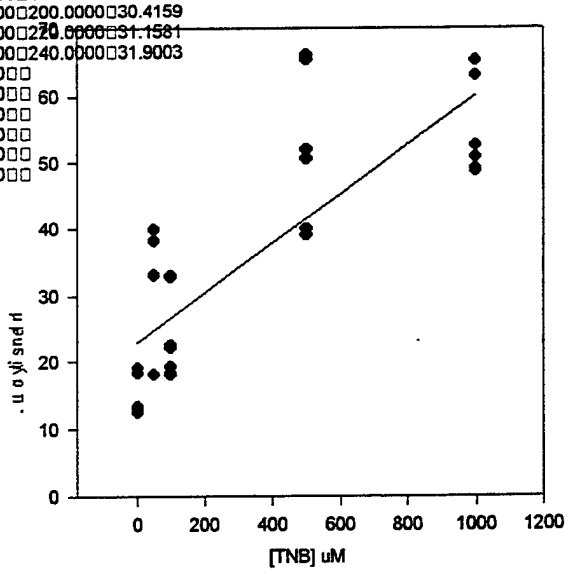
SECTION 6

APPENDIX B

Regression Analysis of EPR data

1. EPR data of Slices.

[TNB]	EPR Intensity
1000.0000	62.8800 Plot 1
1000.0000	65.1400 Order 1
1000.0000	48.7600
1000.0000	52.4100 All curves:
1000.0000	48.9000 Coefficients:
1000.0000	50.6700 b[0] 22.9937
500.0000	50.5600 b[1] 0.0371
500.0000	51.8600 r^2 0.6564
500.0000	38.9900
500.0000	39.9500 Function Values
500.0000	65.3300 x f(x)
500.0000	65.8500 0.0000 22.9937
100.0000	32.6600 20.0000 23.7359
100.0000	32.7900 40.0000 24.4781
100.0000	22.0400 60.0000 25.2203
100.0000	22.3700 80.0000 25.9626
100.0000	18.1500 100.0000 26.7048
100.0000	19.1800 120.0000 27.4470
50.0000	32.9100 140.0000 28.1892
50.0000	33.0400 160.0000 28.9314
50.0000	38.0900 180.0000 29.6737
50.0000	39.8200 200.0000 30.4159
50.0000	18.1200 220.0000 31.1581
50.0000	18.1200 240.0000 31.9003
0.0000	12.6700
0.0000	12.4800
0.0000	12.7600
0.0000	18.2900
0.0000	13.3400
0.0000	19.0800



2. EPR of spin label standards (0-500 uM) run under the same conditions as the slices.